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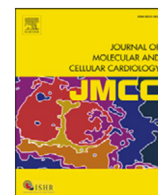

Original article

Combined deep microRNA and mRNA sequencing identifies protective transcriptomal signature of enhanced PI3Kα signaling in cardiac hypertrophy

Kai-Chien Yang [a], Yuan-Chieh Ku [b], Michael Lovett [b], Jeanne M. Nerbonne [a,*]

[a] Department of Developmental Biology, Washington University Medical School, St. Louis, MO 63110, USA
[b] Division of Human Genetics, Department of Genetics, Washington University Medical School, St. Louis, MO 63110, USA

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ABSTRACT

The perturbation of myocardial transcriptome homeostasis is the hallmark of pathological hypertrophy, underlying the maladaptive myocardial remodeling secondary to pathological stresses. Classic and novel therapeutics that provide beneficial effects against pathological remodeling likely impact myocardial transcriptome architecture, including miRNA and mRNA expression profiles. Microarray and PCR-based technologies, although employed extensively, cannot provide adequate sequence coverage or quantitative accuracy to test this hypothesis directly. The goal of this study was to develop and exploit next-generation sequencing approaches for comprehensive and quantitative analyses of myocardial miRNAs and mRNAs to test the hypothesis that augmented phosphoinositide-3-kinase-p110α (PI3Kα) signaling in the setting of pathological hypertrophy provides beneficial effects through remodeling of the myocardial transcriptome signature. In these studies, a molecular and bioinformatic pipeline permitting comprehensive analysis and quantification of myocardial miRNA and mRNA expression with next-generation sequencing was developed and the impact of enhanced PI3Kα signaling on the myocardial transcriptome signature of pressure overload-induced pathological hypertrophy was explored. These analyses identified multiple miRNAs and mRNAs that were abnormally expressed in pathological hypertrophy and partially or completely normalized with increased PI3Kα signaling. Additionally, several novel miRNAs potentially linked to remodeling in cardiac hypertrophy were identified. Additional experiments revealed that increased PI3Kα signaling reduces cardiac fibrosis in pathological hypertrophy through modulating TGF-β signaling and miR-21 expression. In conclusion, using the approach of combined miRNA and mRNA sequencing, we identify the protective transcriptome signature of enhanced PI3Kα signaling in the context of pathological hypertrophy, and demonstrate the regulation of TGF-β/miR-21 by which enhanced PI3Kα signaling protects against cardiac fibrosis.

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1. Introduction

Pathological cardiac hypertrophy, a maladaptive response of the myocardium to increased biomechanical stresses, is associated with increased propensity to develop interstitial fibrosis, heart failure, life-threatening arrhythmias and sudden cardiac death [1,2]. Physiological hypertrophy in response to exercise training, in contrast, is characterized by normal cardiac structure and maintained cardiac function, and is not associated with increased risk of heart failure, arrhythmias or sudden cardiac death [2,3]. Two distinct signaling mechanisms, G-protein coupled receptor (GPCR) signaling [2,4,5] and phosphoinositide-3-kinase-p110α (PI3Kα) signaling [3], have been linked to the development of pathological and physiological hypertrophy, respectively. In addition, it has been demonstrated that augmentation of PI3Kα signaling is beneficial in the setting of pathological hypertrophy and heart failure [6,7], resulting in improved cardiac function and survival. The mechanisms through which enhanced PI3Kα signaling is protective, however, have not been delineated.

A hallmark of pathological hypertrophy is that the myocardial transcriptome landscape, including mRNAs, miRNAs, long noncoding RNAs and other RNA species, is dramatically altered [8,9], suggesting that perturbation of transcriptome homeostasis is critically coupled to the disease process. We hypothesized that augmented PI3Kα signaling provides protective effects by preventing or reversing the changes in myocardial miRNA and mRNA expression associated

Abbreviations: ANF, atrial natriuretic factor; caPI3Kα, constitutively active phosphoinositide-3-kinase p110α; FDR, false discovery rate; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; GO, gene ontology; GPCR, G-protein coupled receptor; LV, Left ventricle; LVH, Left ventricular hypertrophy; LVW/TL, LV weight to tibia length ratio; MI, myocardial infarction; miRNA-Seq, microRNA sequencing; mRNA-Seq, mRNA sequencing; qRT-PCR, quantitative real time polymerase chain reaction; PMMR, sequences per million mapped reads; RPKM, reads per kilobase of exon per million mapped reads; TAC, transverse aortic constriction; WT, wild-type.

* Corresponding author at: Department of Developmental Biology, Washington University Medical School, 660 South Euclid Avenue Box 8103, St. Louis, MO 63110-1093, USA. Tel.: +1 314 362 2564; fax: +1 314 362 7058.
E-mail address: jnerbonne@wustl.edu (J.M. Nerbonne).

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with pathological hypertrophy. Exploiting a molecular and bioinformatic pipeline optimized for transcriptome profiling with next-generation high-throughput sequencing, we examined the impact of increased PI3Kα signaling on myocardial transcriptome structure in the setting of pathological hypertrophy. These analyses identified multiple miRNAs and mRNAs that were abnormally expressed in pathological hypertrophy and normalized with enhanced PI3Kα signaling. TAC-induced pathological remodeling is blunted with enhanced PI3Kα signaling and is associated with the reduced expression of multiple miRNAs linked to cardiac pathology and diseases, including miR-199a-5p, miR-214 and miR-212. In addition, the combined miRNA and mRNA analyses here revealed that increased PI3Kα signaling modulates TGF-β signaling and miR-21 expression, effects that will result in reduced fibrosis. Taken together the data presented here provide new insights into the link between PI3Kα signaling and cardiac miRNA regulation, as well as into the mechanisms through which enhanced PI3Kα signaling mitigates maladaptive remodeling induced by pathological stresses.

2. Methods

An expanded Methods section is available in the Online Data Supplement.

All high-throughput sequencing data have been submitted to the NCBI gene expression and hybridization array data repository (GEO ID: GSE35350) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=pvwltsikocuyglw&acc=GSE35350).

2.1. Experimental animals

Eight to twelve-week-old male WT FVB mice and a transgenic mouse line with cardiac specific expression of constitutively active PI3Kα (caPI3Kα) [10] were subjected to transverse aortic constriction (TAC) to produce pressure overload-induced left ventricular hypertrophy (LVH) [11]. Seven days after surgery, sham-operated and TAC−WT and caPI3Kα animals were examined with echocardiography, followed by collection of cardiac tissues. LV weight to tibia length (LVW/TL) ratios were recorded at the time of tissue collection. The echocardiographic and LVW/TL measurements from all of experimental animals are provided in Supplemental Table S1.

2.2. Small RNA library construction and sequencing

Total RNA from the LV of individual animals was isolated with Trizol (Life Technologies) using described methods [12,13]. The quality and integrity of the RNA samples prepared were confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies). Small RNA libraries were prepared using TrueSeq Small RNA Sample Prep Kits (Illumina) in accordance with the manufacturer's instructions. Briefly, 3′ and 5′ adapters were sequentially ligated to small RNAs (from 1 μg total RNA), followed by a reverse transcription reaction to create single stranded cDNAs, which were then amplified (by PCR) and barcoded using a common primer and a primer containing unique six-base index sequence. The amplified libraries were size-selected/gel-purified and quantified using Qubit dsDNA HS Assay kit (Life Technologies). Six to eight barcoded libraries were pooled in equimolar (10 nmol/L) amounts and diluted to 8 pmol/L for cluster formation on a single flow cell lane, followed by single-end sequencing on an Illumina HiSeq 2000 sequencer. A total of 15 small RNA libraries were sequenced (see results) using two HiSeq2000 flow lanes, which provides >5× sequence coverage of the mouse cardiac miRNome for each sample, based on the estimation of 100–200 million sequence reads generated per lane.

2.3. Small RNA sequencing data analyses

Sequencing data from samples pooled in the same flow cell lane were separated (demultiplexed) using CASAVA 1.6 software (Illumina). A total of 165.5 million reads were obtained from sequencing 15 small RNA libraries (Table 1). The sequence reads were analyzed using the miRanalyzer program [14,15], where the raw sequencing data were transformed and filtered to keep only sequences containing 17–26 bases. Filtered reads were then successively mapped (using Bowtie) [16] to: (1) the miRBase [17] v.18 mouse database (allowing up to two mismatches), to detect known miRNAs; (2) the RefSeq and Rfam database, to detect contamination from other RNA species; and (3) the mouse genome to detect potential novel miRNAs. The sequences matching known miRNAs were clustered and counted; the read counts of each of the known miRNAs were then normalized to the total counts of sequences mapped to the miRBase v.18 database and presented as PMMR (sequences per million mapped reads).

2.4. Discovery of novel miRNAs

Sequence reads that are counterparts of known mature miRNAs, but that have not been previously reported in the miRBase database, were identified and defined as novel miRNA-star (miRNA*). Sequence reads that did not match known miRNAs or known transcripts from the RefSeq and Rfam databases were mapped against the mouse genome (M37). Once aligned, sequence reads that shared characteristics of miRNAs were identified and clustered using the computational algorithms provided by miRAnalyzer [14]. These clusters were further filtered to exclude contamination from repetitive sequences and from mRNA or noncoding RNA degradation products. Only clusters with a read depth ≥100 reads were chosen for further analyses. The filtered candidate miRNAs and their precursors were

Table 1
Summary of miRNA- and mRNA-Seq read counts and mapping results.

miRNA-Seq read counts	WT sham (n=4)	WT TAC (n=5)	caPI3Kα sham (n=3)	caPI3Kα TAC (n=3)	Total
Total	45,901,044	49,701,319	37,441,052	32,452,959	165,496,374
Mapped	41,986,529 (91.5)	45,593,199 (91.7%)	34,304,088 (91.6%)	30,735,651 (94.7%)	152,619,467 (92.2%)
Unmapped	3,914,515 (8.5%)	4,108,120 (8.3%)	3,136,964 (8.4%)	1,717,308 (5.3%)	12,876,907 (7.8%)
Known miRNAs	22,981,918 (50.1%)	27,443,912 (55.2%)	18,360,999 (49.0%)	19,354,436 (59.6%)	88,141,265 (53.3%)
Putative miRNAs	2,514,756 (5.5%)	2,564,454 (5.2%)	2,696,651 (7.2%)	2,444,635 (7.5%)	10,220,496 (6.2%)
Mapped to other RNA species	2,968,104 (6.5%)	2,942,701 (5.9%)	2,950,463 (7.9%)	2,282,158 (7.0%)	11,143,426 (6.7%)
Mapped to genome, unannotated	13,521,751 (29.5%)	12,642,132 (25.4%)	10,295,975 (27.5%)	6,654,422 (20.5%)	43,114,280 (26.1%)
mRNA-Seq read counts	**WT sham (n=4)**	**WT+TAC (n=3)**	**caPI3Kα sham (n=2)**	**caPI3Kα+TAC (n=2)**	**Total**
Total	47,913,934	45,178,741	84,373,729	65,384,703	242,851,107
Mapped	40,884,751 (85.3%)	38,297,928 (84.8%)	70,954,141 (84.1%)	55,108,361 (84.3%)	205,245,181 (84.5%)
Unmapped	7,029,183 (14.7%)	6,880,813 (15.2%)	13,419,588 (15.9%)	10,276,342 (15.7%)	37,605,926 (15.5%)
Mapped to exon regions	26,279,745 (54.8%)	26,838,716 (59.4%)	48,917,894 (58.0%)	37,761,525 (57.8%)	139,797,880 (57.6%)

TAC: trans-thoracic aortic constriction.

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